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**IMPACT OF POLYCHLORINATED BIPHENYL CONGENER 126
(PCB 126) ON THE IMMUNE FUNCTION OF AGED AND
JUVENILE JAPANESE MEDAKA (*Oryzias latipes*)**

By
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October, 2001

A thesis in the Department of Biology submitted to the faculty of Graduate School of
Arts and Sciences in partial fulfillment of the requirements
for the degree of Master of Science
at New York University

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Objectives:

To determine if the Japanese medaka immune system is sufficiently sensitive for predicting PCB-induced immunotoxicity, and to examine the role of host age and post-exposure duration on the impact of PCB-induced immunodysfunction.

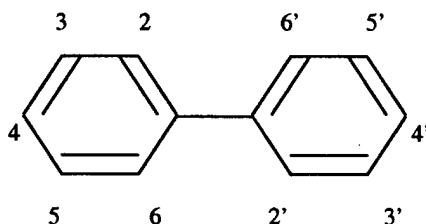
Hypothesis:

It is hypothesized that: (1) Japanese medaka are sensitive to the immunomodulating effects of a coplanar PCB congener; (2) aged medaka are more sensitive to the immunomodulating effects of PCBs than their juvenile counterparts due to age-related compromise of the immune system; and, (3) a longer post-exposure duration will intensify PCB-induced immunotoxicity.

Introduction:

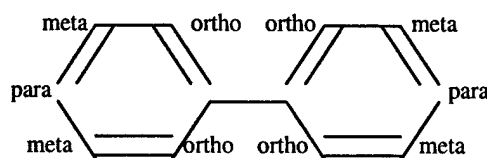
Polychlorinated biphenyls (PCBs) exist today as a major contaminant in water resources worldwide. Produced in the United States under the trade name Aroclor, PCBs were synthesized for use as insulating fluids, plasticizers, and various industrial applications (Swain, 1983; Safe et al., 1987). Since their recognition as an environmental hazard in the 1960's, PCB production in the US ceased in the late 1970's. Nevertheless, PCBs continue to persist in the environment due to their high lipid solubility and, subsequently, bioaccumulate throughout the food chain.

Polychlorinated biphenyls are produced by the substitution of chlorine molecules on biphenyl rings; 209 different substitution patterns (i.e. congeners) exist, varying in the number and position of chlorine molecules.



The number of chlorine molecules determines the solubility and, therefore, persistence of each congener in water; as the chlorine content of the congener increases, the less water-soluble the congener becomes (Swain, 1983). Furthermore, PCBs can form strong bonds with particulate matter, leading to increased concentration of these contaminants in soils and sediments with estimated half-lives of 8 – 15 years (Swain, 1983). The chemical stability of PCBs further adds to the persistence of these contaminants. For example, PCBs fail to react with acids/bases or undergo hydrolysis, chemical oxidation, or photodegradation. They are also thermally stable and resistant to many chemical agents (Swain, 1983).

Polychlorinated biphenyl congeners are divided into three classes based upon the positioning of the chlorine molecules: coplanar, mono-ortho coplanar, and non-coplanar (McFarland and Clarke, 1989). Chlorine substitution at the ortho, meta and/or para positions determines which class a particular congener belongs to.



Mono-ortho and non-coplanar congeners have at least one chlorine substitution at an ortho position, whereas coplanar congeners do not. Coplanar congeners appear to have the greatest potential for toxicity. McFarland and Clarke (1989) reported that congeners with chlorine substitution at the para, at least two meta, and no ortho positions are the most toxicologically active. Furthermore, coplanar PCB congeners have the greatest affinity for the Ah receptor (similar to that of dioxin), while non-coplanar congeners have the lowest affinity (Stack et al., 1999).

Exposure of mammals to PCBs (as well as other species high up in the food chain) is most likely due to the consumption of fish and other aquatic organisms living in PCB-contaminated environments. Upon entering the GI system of mammals, PCBs can be absorbed and distributed to tissues throughout the body. Tissue distribution is initially dependent upon blood flow; tissues with high blood flow (i.e. liver and muscle) contain the highest PCB concentrations (Matthews, 1983; Sipes and Schnellmann, 1987). However, PCBs will ultimately become concentrated in tissues high in lipid content where they can remain indefinitely (i.e. adipose). Polychlorinated biphenyl congeners can undergo metabolism (Matthews, 1983; Sipes and Schnellmann, 1987). However, the rate and extent to which PCBs are biotransformed is dependent upon the individual properties of the congener. For example, the number and positioning of chlorine molecules are important factors in determining metabolism of each particular congener. It appears that congeners containing a high number of chlorine molecules are less rapidly metabolized and excreted than congeners with fewer chlorine substitutions (Sipes and Schnellmann, 1987). Another factor affecting mammalian metabolism of PCB

congeners is the presence or absence of adjacent unsubstituted carbon molecules. In the absence of adjacent unsubstituted carbons, metabolism occurs at a much slower rate because the chlorine molecules sterically block the enzymatic activity that biotransforms the compound into polar (more excretable) products (Matthews, 1983). It has been reported that the products of PCB metabolism excreted in the urine are polar metabolites, whereas the non-polar, parent compound remains in host tissues. Although the rate of metabolism of PCBs may differ between mammalian species (dogs > rats/mice > monkeys), overall mechanisms appear to be similar (Matthews, 1983). While mammals can readily excrete some PCB congeners, the same is not true for many fish species. Fish metabolize PCBs slowly despite the number of chlorine molecules present, thus, biotransformation and excretion is notably slower in fish than in mammals (Lech and Peterson, 1983).

Polychlorinated biphenyls have a plethora of toxic effects. For example, exposure to PCBs may produce injury to the hepatic, immune, nervous, and dermatological systems, as well as impair reproductive, gastrointestinal, and respiratory function (Hansen, 1987; Parkinson and Safe, 1987). Moreover, PCB exposure has also been shown to have mutagenic and carcinogenic effects (Hayes, 1987; Parkinson and Safe, 1987). The degree to which any of these effects are observed, however, is dependent upon host species, strain, gender, and age (Safe, 1986).

Negative impacts of PCB exposure on the human population have been documented. In 1968, more than 1600 people in Japan became ill after ingesting PCB-contaminated rice

bran oil. More than ten years later in 1979, 2000 people were poisoned in Taiwan, again from the consumption of PCB-contaminated rice bran oil; these occurrences were termed "Yusho" and "Yu-cheng", respectively. Affected individuals from both incidents showed signs of immunosuppression (Lee and Chang, 1985).

Effect of PCBs on the immune system has only been modestly examined despite studies in laboratory animals suggesting that the immune system is a sensitive target for PCB-induced toxicity (Tryphonas, 1995). The mechanism of action for PCB-induced immunotoxicity may include binding of the Ah receptor (Tryphonas, 1995); affinity for this receptor appears to be a determining factor in the production of immunotoxic effects (Tryphonas, 1995).

To date, studies investigating PCB-induced immunotoxicity have primarily concerned effects upon mammalian species. Effects of PCB exposure upon humoral-mediated immune function appear to be most frequently reported. For example, PCBs, either as a mixture or individual congeners, have been shown to suppress the plaque forming cell (PFC) response in mice (Loose et al., 1978; Lubet et al., 1986; Davis and Safe, 1990; Harper et al., 1995). Many of these studies involved mice genetically engineered to be either Ah-responsive (Ah+) or non-responsive (Ah-). It was shown that mice that are Ah- do not experience the same PCB-induced suppression of PFC numbers as mice that are Ah+. For example, B6C3F₁N (Ah+) and C57BL/6N (Ah+) mice injected intraperitoneal (i.p.) with Aroclor 1254 (at concentrations ranging from 250 – 750 mg/kg) and examined after 5 d, exhibited significant reductions in PFC numbers compared to controls;

DBA/2N (Ah-) mice failed to demonstrate any significant effects (compared to controls) (Lubet et al., 1986). This data indicates that the Ah receptor is required for PCB-induced suppression of humoral immunity in mice (Lubet et al., 1986). Furthermore, *in vitro* studies with mice splenocytes showed that PCB mixtures and/or individual congeners inhibited lipopolysaccharide (LPS)-induced splenocyte proliferation (Yoo et al., 1997; Stack et al., 1999). Aroclors and certain non-coplanar congeners significantly inhibited LPS-induced splenocyte proliferation while planar congeners produced no significant effects on the same endpoint, suggesting that this response is not Ah receptor-dependent.

While results appear inconsistent across mammalian species, effects of PCBs upon cell-mediated immunity have also been reported. For example, Smialowicz et al. (1989) reported an enhanced lymphoproliferative response to the T-cell mitogen phytohemagglutinin (PHA) in rats dosed daily by oral gavage for 15 weeks with Aroclor 1254 (25 mg/kg BW). In contrast, infant rhesus (*Macaca mulatta*) and cynomolgus (*M. fascicularis*) monkeys fed a mixture of PCB congeners (7.5 µg/kg BW) daily for 20 weeks showed no significant differences in lymphoproliferative response to T-cell mitogens between control and PCB-treatment groups (Arnold et al., 1999). Similarly, no effects were observed on mitogen-stimulated B-cell proliferation by splenocytes recovered from mice 15 d post-injection with Aroclor 1254 (up to 1000 mg/kg BW); at 8 d post-treatment, administration of 500 mg Aroclor 1254/kg BW slightly enhanced T-cell proliferation in response to mitogen (Lubet et al., 1986). Whether these observed inconsistencies were attributable to species variability, administered dose, route of exposure, and/or PCB formulation, is still unknown.

Field studies examining cell-mediated immune function in feral populations living in PCB-contaminated environments have also demonstrated impaired immune responses. For example, young caspian terns (*Sterna caspia*) living in an organochlorine-contaminated environment in Saginaw Bay (Lake Huron) exhibited suppressed T-cell function, as measured by the PHA delayed hypersensitivity skin test (Grasman and Fox, 2001). Moreover, human populations inadvertently exposed to PCBs (Yu-cheng patients) have also demonstrated immunodysfunction (Lee and Chang, 1985). Upon examination, the delayed hypersensitivity response to streptokinase and streptodornase was positive in only 40% of Yu-cheng patients, compared to an 80% response in healthy individuals. Epidemiological studies involving breast-feeding mothers have found that infants whose mothers consumed large amounts of Great Lakes fish had higher occurrences of microbial infections than those infants whose mothers did not consume fish (Tryphonas, 1995).

Effects of PCB upon host resistance have also been examined in mammals. Reduced resistance to infection with the gram-negative bacteria *Listeria monocytogenes* has been observed in mice 5 d following i.p. injection of 500 mg/kg BW Aroclor 1254 (Lubet et al., 1986). Dietary administration of 167 ppm Aroclor 1242 for 6 weeks significantly increased mice sensitivity to infection with *Salmonella typhosa* (Loose et al., 1978); PCB-treated mice also exhibited a decreased mean survival time (compared to control) following challenge with *Plasmodium berghei* NYU-2. Furthermore, mice fed a PCB diet (400 µg/g) for 21 d and subsequently exposed intranasally to influenza virus demonstrated significantly greater mortality than controls (Imanishi et al., 1984). In the

same study, mice fed diets containing 200 or 400 µg PCB/g for 21 d exhibited greater mortality rates (compared to control) following i.p. injection of *Staphylococcus aureus*. These observations provide further support that exposure to PCBs can impair immune function and host resistance.

Nonspecific immunity has also been reported to be impacted by PCB exposure. Ganey et al. (1993) reported that rat polymorphonuclear leukocytes (PMNs) treated *in vitro* with Aroclor 1242 demonstrated increased basal and PMA-stimulated levels of superoxide (O_2^-) production over a 40 min incubation period. Effects of two PCB congeners on PMN function were also examined. Overall, results demonstrated that while a congener with strong affinity for the Ah receptor failed to affect PMN function, one with less affinity produced effects on PMN function similar to those observed for Aroclor 1242 (Ganey et al., 1993). In another study, rat PMN treated for 30 min *in vitro* with coplanar PCB congeners (10 or 50 µM) had no effect upon O_2^- production (Brown et al., 1998).

While an extensive amount of toxicity data is available for mammals, few studies have investigated the impact of PCB exposure on fish despite the fact that these species can be chronically exposed in their aquatic environment. For example, the Great Lakes, which comprise 20% of the worlds freshwater aquatic supply (Swain, 1983), have been shown to contain PCB concentrations in open water ranging from 0.5-5 ng/L (Eisenreich and Johnson, 1983). Resident organisms accumulate these contaminants in their body tissues (i.e. adipose tissue), which subsequently become biomagnified as they are consumed by species higher in the food chain. Fish found within the five Great Lakes including lake

trout, siscowet trout, yellow perch, and coho salmon have been found to contain mean body burdens of PCBs in the range of 2 – 20 mg PCB/kg, depending upon the fish species and particular lake examined (Swain, 1983).

From the few studies that have examined effects of PCB exposure on fish immune defense mechanisms, results have been inconsistent. As previously described in mammals, PCB exposure appears to suppress the PFC response of exposed feral fish populations. Arkoosh et al. (1994) reported a decreased PFC response after only a single i.p. injection of Aroclor 1254 (54 mg/kg BW) into juvenile chinook salmon (*Oncorhynchus tshawytscha*). In this study, both primary and secondary PFC responses to T-dependent and -independent antigens by anterior kidney and splenic leukocytes were examined. Polychlorinated biphenyl-exposed salmon demonstrated suppressed primary and secondary PFC responses (in both cell populations) to the T-independent antigen, as well as a suppressed secondary response to the T-dependent antigen. In contrast, a study by Cleland et al. (1988) failed to show any significant effects on the T-dependent PFC response in rainbow trout (*Salmo gairdneri*) following dietary exposure for 12 mo to 3, 30 or 300 ppm Aroclor 1254; weight loss and hepatomegaly were observed in trout fed the highest Aroclor concentration (i.e. 300 ppm).

Nonspecific (innate) immune responses in fish, such as phagocyte-mediated O_2^- production, appear to be sensitive to PCB exposure. For example, channel catfish (*Ictalurus punctatus*) that received a single i.p. injection of either 0.01 or 1.0 PCB 126 mg/kg BW demonstrated suppressed kidney phagocyte oxidative burst activity

(compared to control) for up to 14 d post-PCB exposure (Regala et al., 2001). In a similar study using identical PCB 126 concentrations, Rice and Schlenk (1995) reported reductions in oxidative burst activity by channel catfish kidney phagocytes beginning at 3-, and persisting for up to 7-, d post-PCB 126 injection.

Despite species variability, evidence is accumulating that exposure to PCBs produces immune dysfunction in fish; inconsistencies in the literature are likely due to the use of different fish species. Because the immune system has a central role in maintaining overall "good health", determining the impact of xenobiotics is an important goal. Polychlorinated biphenyls, ubiquitous contaminants in the aquatic environment, pose a threat not only to directly-exposed species, but also to those exposed indirectly due to biomagnification of these pollutants. For example, people residing in the Great Lakes region may be at risk for adverse health effects induced by PCB exposure via the consumption of fish and/or drinking water (Tryphonas, 1995). Therefore, investigations concerning effects of PCB exposure on the immune system are warranted.

In the present study, Japanese medaka (*Oryzias latipes*) were used as a model to determine sensitivity of the fish immune system to a particular PCB congener and to assess whether responses were consistent with those observed in mammals. Furthermore, host age and post-exposure duration were examined as possible variables influencing the impact of PCB 126-induced immunotoxicity. PCB 126 was chosen for study because due to its coplanar structure, it is reportedly the most potent PCB Ah receptor agonist (Twaroski et al., 2001), with an affinity for the Ah receptor one-tenth that of 2,3,7,8-

tetrachlorodibenzo-p-dioxin (TCDD) (Rice and Roszell, 1998). McFarland and Clarke (1989) identified PCB 126 as a "high priority congener", based upon its toxic potential, environmental occurrence, and abundance in animal tissues. Although only found in the parts-per-trillion (ppt) range in environmental samples, the potency of this congener calls for special examination (McFarland and Clarke, 1989).

Materials and Methods:

Chemical:

PCB congener 126 (3,3',4,4',5-pentachlorobiphenyl) was purchased from AccuStandard Inc. (New Haven, CT). Stock solution of the congener was prepared with 0.9 mg PCB 126/ 9 ml corn oil. The final solution was heated overnight in a 60°C waterbath to allow dissolution of the congener.

Fish:

Japanese medaka (*Oryzias latipes*), at either 4 – 6- (juvenile) or 12 – 15-mo of age (aged), were supplied by the United States Army Center for Environmental Health Research (USACEHR, Fort Detrick, MD). Fish were maintained in 10 gallon glass aquaria at $25^{\circ}\pm 2^{\circ}\text{C}$ and acclimated for at least one week prior to use. Medaka were fed once per day (excluding days that fish received i.p. injections or were sacrificed) a diet of TetraMin flakes that were ground and mixed with distilled water. Fish were maintained on a 16/8 h light/dark cycle. Water conditions, including temperature, pH, and dissolved oxygen and ammonia, were measured throughout each experiment using colorimetric kits (LaMotte, Chestertown, MD). Tank water was changed on a weekly basis.

Experimental Design:

Stock solution was diluted in corn oil in order to achieve final PCB 126 concentrations of 0.01, 0.1 and 1.0 $\mu\text{g/g}$ BW. The PCB dose of 0.1 $\mu\text{g/g}$ BW was used only for range-finding mortality studies. In all cases, fish were individually weighed in order to determine the exact amount (μl) of solution needed to achieve the desired PCB concentration. The dose was calculated such that a 0.5 μg fish received 20 μl of either the PCB congener or vehicle control. Fish received a single i.p. injection of either PCB 126 or the corn-oil vehicle control. Polychlorinated biphenyl and corn-oil injections utilized 30½ G syringes. Each 10 gallon tank contained between 30-50 and 10-30 juvenile and aged fish, respectively. Fish, injected with either corn-oil or 0.01, 0.1, or 1.0 μg PCB126/g BW were observed daily for 14 d to assess any changes in host morbidity/mortality.

Fish were sacrificed by decapitation at either 3 or 14 d post-injection and the kidney and/or liver aseptically removed. Kidneys, pooled within a given treatment group, were maintained in 3ml of supplemented fish physiological saline (FPS + 1% glucose) until needed for the designated immune assay (no longer than 24 h). Livers were placed into cold 0.5ml centrifuge tubes, frozen at -180°C in liquid nitrogen, and stored at -70°C until used; between 3 – 5 livers per treatment group were individually stored for determining protein content and CYP1A enzyme induction. Remaining livers from each treatment group were pooled and stored under the same conditions in the event that an individual liver sample did not provide enough protein necessary for the Enzyme-linked Immunosorbant Assay (ELISA).

Ex Vivo Assays:

Intracellular O₂⁻ Production

Intracellular O₂⁻ production was examined as a measure of PCB-induced changes in the nonspecific immune response. Kidneys from each exposure group were homogenized in supplemented FPS (using glass/glass homogenizers) and cell counts determined by hemacytometer counting. Cell concentrations were adjusted to 6x10⁶ cells/ml in supplemented L-15 media [Leibovitz Medium, 5% fetal bovine serum (FBS), 1.0% penicillin/streptomycin, 0.5% L-Glutamine, 15mM HEPES buffer]. Cells (in 100 µl volumes) were added in quadruplicate to four rows of a 96-well microtitre plate (for a total of 16 wells) and incubated (at 30°C) for 90 min in a humidified incubator (to provide ample time for cell attachment). During incubation, four reaction mixtures were prepared using the following reagents: Hank's Balanced Salt Solution (HBSS; GIBCO); nitroblue tetrazolium (NBT; 1 mg/ml stock prepared in HBSS); superoxide dismutase (SOD; 300 µg/ml HBSS); and phorbol 12-myristate 13-acetate [PMA; 1 µg/ml dimethyl sulfoxide (DMSO) diluted to a final concentration of 10 µg/ml HBSS]. Different combinations of the above reagents were used to prepare each reaction mixture.

Reagent	Tube 1	Tube 2	Tube 3	Tube 4
NBT	1.6 ml	1.6 ml	1.6 ml	1.6 ml
SOD	---	0.2 ml	---	0.2 ml
PMA	---	---	0.2 ml	0.2 ml
HBSS	0.4 ml	0.2 ml	0.2 ml	---

Following incubation, covering medium containing detached cells was removed from each well, pooled within individual treatment groups, and cell counts determined by hemacytometer counting (later used to determine the actual percentage of attached cells). Reaction mixtures were added in 100 μ l/well volumes in the following manner: tubes 1 – 4 were added to the first, second, third, and fourth column of wells, respectively. The plate was then incubated (at 30°C) for 60 min. Following incubation, covering medium was removed from each well and discarded. Each well was then washed three times with 70% methanol (100 μ l/well); the first methanol wash remained in the wells for 5-10 min. When wells were completely dry (approximately 10-15 min after the third wash), 120 μ l of potassium hydroxide (KOH, 2M) was added to each well. The plate was then mixed at 300 rpm for 10 min using an Orbital Shaker (Fisher Scientific). Dimethyl sulfoxide was then added to each well (140 μ l/well), thoroughly mixed, and the absorbance measured spectrophotometrically at 630 nm in a microtitre plate reader. Average absorbance per treatment group was calculated for each column of wells. Final optical density (OD) was calculated by subtracting the average absorbance of column 1 from the average absorbance of column 2 (unstimulated cells) and then subtracting the average absorbance of column 3 from the average absorbance of column 4 (PMA-stimulated cells). The resulting OD values were multiplied by 15.87 to yield final nmoles of O_2^- (Pick and Mizel, 1981). Although 6×10^5 cells were added to each well, not all of the cells attached. Thus, the number of detached cells counted within a given treatment group were used to determine the actual percentage of attached cells. The OD values calculated for unstimulated and PMA-stimulated cells were then divided by the percentage of attached cells to determine final nmoles of O_2^- (per 6×10^5 cells/ 60 min) (Zelikoff et al., 1996).

Plaque Forming Cell Assay

The PFC assay, used to determine effects of PCB 126 on humoral immunity, was performed using a modified version of the Jerne plaque assay (Beaman et al., 1999). Three or 14 d after exposure to either PCB 126 or vehicle, fish were immunized via i.p. injection (using 28 ½ G insulin syringe) with a 10% solution of sheep red blood cells [SRBCs, suspended in Dulbecco's Phosphate-Buffered Saline (DPBS; GIBCO), were washed three times in DPBS 24 h prior to use and again on the day of injection]. Fifteen-month-old fish were administered a single 20 µl injection of SRBCs and sacrificed 11 d later; 4-month-old fish were administered a single 10 µl injection of SRBCs and sacrificed after 7 d. Upon sacrifice, kidneys were removed, pooled within a given exposure group, and maintained in supplemented FPS for no longer than 24 h. Pooled kidneys from within a single exposure group were homogenized and cell concentrations adjusted to 20×10^6 cells/ml in supplemented L-15 medium [Leibovitz Medium, 5% fetal bovine serum (FBS), 1.0% penicillin/streptomycin, 0.5% L-Glutamine, 15mM HEPES buffer].

An agarose solution was prepared using 150 mg agarose (Sigma) per 10 ml DPBS. The solution was brought to a rapid boil (to dissolve the agarose) and maintained in a 45°C waterbath until needed. Dextran (10.7 mg; Sigma) was added to the agarose solution and dissolved by gently inverting the tube several times. Culture tubes (3 ml), maintained at 45°C, were used to hold the plaquing mixtures (typically 3-5 tubes per treatment group, depending upon the number of kidney cells available) that contained: 100 µl of agarose solution, 2X L-15 media and cells, and 200 µl of SRBCs. Tubes were then mixed for approximately 20 sec using a vortex mixer on low speed. The contents of each tube were

poured evenly over a microscope slide and allowed to solidify for 3 - 4 min. Upon solidification, slides were transferred to a humidified chamber and incubated for 4 h (at 30°C). Following incubation, 900 µl of guinea pig complement (diluted 1:30 in PBS) was added to each slide. Humidified chambers were returned to the incubator and after 18 h plaques were counted using a dissecting microscope. Plaques were determined by the presence of a B-cell in the center of a clear circular area within a lawn of SRBCs. Final PFC numbers were determined for each treatment group by calculating the average number of plaques from all of the slides within a given treatment group (typically 3 - 5 slides per treatment group). Values were expressed as a percentage of controls.

Microsome Preparation

Livers were used to measure protein concentration and determine CYP1A activity by ELISA (Nilsen et al., 1998). Either pooled or individual liver samples from within each treatment group were homogenized in a microsome homogenization buffer [0.1M sodium phosphate, pH 7.4, 0.15M KCl, 1mM EDTA, 1mM dithiothreitol (DTT)]. Samples were then centrifuged for 20 min at 10,000 rpm. After centrifugation, supernatants were removed, pipetted into cold 50 ml polystyrene centrifuge tubes and ultracentrifuged for 90 min (at 28,000 rpm). Supernatants (containing the cytosol) were discarded and the remaining pellets (containing the microsomes) were re-suspended in a microsome re-suspension buffer [0.1M sodium phosphate, pH 7.4, 0.15M KCl, 1mM DTT, 20% (v/v) glycerol].

Protein Determination

Protein content of each microsomal sample was determined from a standard curve prepared with bovine serum albumin (BSA); a series of eight two-fold dilutions were prepared. Five μ l of each BSA standard, a blank (i.e. PBS alone), and microsomal samples were added in triplicate to individual wells of a 96-well microtitre plate. Reagent A (25 μ l/well) and reagent B (200 μ l/well), solutions from a protein assay kit (BioRad), were added. The plate was then placed into a microtitre plate reader set to "shake" for 5 sec. After 15 min, absorbance was measured spectrophotometrically at 750 nm. Average absorbance was determined for each standard and microsomal sample. Using the computer program Excel, protein concentration (mg/ml) of each standard (x-axis) was plotted against the average absorbance (y-axis) and the best-fit line determined by regression analysis. Protein concentration (mg/ml) was determined from the average absorbance of each microsomal sample.

Enzyme-Linked Immunosorbant Assay

Microsomal samples were diluted to 10 μ g protein/100 μ l coating buffer (50mM carbonate/ bicarbonate) and kept on ice until needed. One-hundred μ l of the prepared solution was added in triplicate to wells of an Immulon 96-well microtitre plate (Dynatech Laboratories, Chantilly, VA); coating buffer, added to all empty wells, served as controls. The plate was then covered with parafilm and stored overnight at 4°C. Twenty-four h later, wells were washed three times with 200 μ l Tween-20 in PBS (TPBS, 0.05%); the final wash remained in the wells for 5 min. Blocking solution (2% BSA in PBS) was added to each well in 200 μ l volumes. The plate was then covered

with parafilm and incubated at room temperature (RT) for 60 min. Following incubation, wells were washed with TPBS, 100 µl primary antibody (Ab) solution (monoclonal Ab C10-7 raised against rainbow trout CYP1A1 peptide, Rice et al., 1998) added to each well, and the plate incubated (at 37°C) for 60 min. Wells were then washed with TPBS, 100 µl secondary Ab solution (anti-mouse IgG Alkaline Phosphatase Conjugate; 1:1000 in 1% blocking solution; Sigma) added, and after a 60 min incubation (at RT) wells washed an additional five times with TPBS. The substrate solution, p-Nitrophenyl Phosphate (p-NPP; Sigma), was added in 100 µl volumes and the plate incubated (at RT) for an additional 30 min. Following incubation, 50 µl NaOH (3N) was added to each well and the plate shaken for 3 sec in a microtitre plate reader. Absorbance of each well was determined spectrophotometrically at 405 nm. Average absorbance was calculated for each microsomal sample and used to determine the fold-induction of CYP1A in PCB-treated fish over controls.

Statistics:

Statistical analyses was performed to determine differences within and between treatment groups. Data was analyzed by one-way analysis of variance (ANOVA), followed by Fisher's post-hoc testing when necessary. Significant differences between and within control and experimental groups were accepted at $p < 0.05$.

Results:

Results from pilot studies (conducted prior to actual experiments) demonstrated that exposure to PCB 126 at doses of 0.01, 0.1 and 1.0 µg/g BW had no significant effect

upon medaka survival for up to 14 d post-injection (Figure 1). Furthermore, fish mortality determined from each individual experiment demonstrated that there were no significant differences between PCB-treatment groups and controls throughout the study duration (Figure 2). In addition, figures 3A and B demonstrate no significant effects of PCB 126 on kidney cell numbers in either age group.

CYP1A Activity

3 d Post-PCB Exposure

As an indicator of PCB exposure, liver CYP1A activity was measured in both juvenile and aged medaka (Figures 4A and B). Injection with the highest PCB dose significantly increased CYP1A activity in both age groups 3 d post-exposure (Figure 4A). While no effects were observed on CYP1A activity at the lower PCB dose, juvenile fish treated with the highest PCB dose demonstrated a 1.4 fold-increase in CYP1A induction above control and 0.01 µg PCB/g BW treated fish. Aged medaka treated with 0.01 µg PCB/g BW demonstrated CYP1A activity similar to that observed in control fish 3 d post-PCB exposure. However, treatment of 15-mo-old fish with 1.0 µg PCB/g BW significantly increased CYP1A induction 1.5 fold above that observed in age-matched controls and fish treated with the lower PCB dose.

14 d Post-PCB Exposure

Figure 4B shows the effects of PCB on CYP1A activity 14 d post-injection. Juvenile fish injected with the lower PCB dose demonstrated CYP1A activity similar to that observed in exposed fish examined 3 d post-exposure (Figures 4A and B). While exposure to 0.01

$\mu\text{g PCB/g BW}$ had no effect upon CYP1A induction in either age group (compared to control), juvenile fish injected with $1.0 \mu\text{g PCB/g BW}$ demonstrated a significant 2.3 fold-induction in CYP1A activity (over control and $0.01 \mu\text{g PCB/g BW}$ -treated fish). Although aged medaka exposed to the higher PCB dose demonstrated a less dramatic increase in CYP1A induction than juvenile fish, induction was still 1.8-fold above control.

Superoxide Production

Unstimulated O_2^- Production 3 d Post-PCB Exposure

In general, O_2^- production by unstimulated phagocytes from juvenile medaka was greater than that produced in aged fish (Figure 5A); O_2^- production by phagocytes from juvenile fish treated with $0.01 \mu\text{g PCB/g BW}$ was significantly greater than that produced by aged fish treated with the same PCB dose. Exposure to the higher PCB concentration had no significant effects on unstimulated O_2^- production in either age group (compared to age-matched vehicle-injected controls).

Unstimulated O_2^- Production 14 d Post-PCB Exposure

At 14 d post-exposure, phagocytes from aged control and PCB-treated medaka produced greater amounts of unstimulated O_2^- than their juvenile counterparts (Figure 5B); production of O_2^- by juvenile fish in the low-PCB treatment group was significantly lower than that produced by exposed 15 - mo-old medaka. In contrast to that observed 3 d post-PCB exposure, phagocytes from 4 - mo-old medaka exposed to the highest PCB concentration produced significantly greater quantities of O_2^- than that produced by

either the age-matched control or fish treated with 0.01 μg PCB/g BW. Phagocytes from aged medaka exposed to 1.0 μg PCB/g BW also produced significantly greater amounts of O_2^- compared to the vehicle control.

Stimulated O_2^- Production 3 d Post-PCB Exposure

PMA-stimulated O_2^- production in juvenile control fish (i.e., 4.92 nmoles) was significantly greater than that produced by their aged counterparts (i.e., 2.43 nmoles) (Figure 6A). Exposure of juvenile medaka to 0.01 and 1.0 μg PCB/g BW significantly depressed PMA-stimulated O_2^- production compared to controls, although no dose-response was observed. Phagocytes from aged fish exposed to 0.01 μg PCB/g BW produced increased amounts of PMA-stimulated O_2^- compared to controls; similar effects were not observed in aged fish at the higher PCB concentration.

Stimulated O_2^- Production 14 d Post-PCB Exposure

Phagocytes from aged control fish produced significantly greater amounts of PMA-stimulated O_2^- (i.e., 4.07 nmoles) than their juvenile counterpart (i.e., 1.66 nmoles) (Figure 6B). Phagocytes recovered from juvenile fish treated with 1.0 μg PCB/g BW produced significantly greater quantities of PMA-stimulated O_2^- than controls or fish exposed to 0.01 μg PCB/g BW. No significant effects of either PCB concentration were observed on PMA-stimulated O_2^- production by phagocytes from aged medaka (compared to age-matched controls).

Plaque Forming Cell Numbers

3 d Post-PCB Exposure

Three days following injection with 1.0 μg PCB/g BW, PFC numbers were significantly suppressed in both aged and juvenile medaka (68 and 53 percent of control, respectively) (Figure 7A). Exposure of either age group to the lower PCB concentration had no effect upon PFC numbers.

14 d Post-PCB Exposure

As post-exposure duration increased from 3 to 14 d, the impact of PCB 126 on PFC numbers became more divergent between the age groups (Figure 7B). Although no dose-response was apparent, both PCB concentrations significantly depressed PFC numbers in juvenile fish. Aged medaka demonstrated significantly depressed PFC numbers (compared to control and 0.01 μg PCB/g BW-treated fish), but only at the highest PCB concentration.

Discussion:

Results from this study have demonstrated that fish (specifically Japanese medaka) are sensitive to the immune-altering effects of PCBs. Moreover, age at the time of exposure and post-exposure duration are important factors in determining impact of PCB 126 upon the immune system.

Hepatic CYP1A Induction

A relationship between PCBs, the Ah receptor, CYP1A induction, and immunotoxicity has been reported in mice (Silkworth et al., 1984). This relationship consists of PCBs binding to the Ah receptor, leading to induction of CYP isozymes and, ultimately, to immunotoxicity. PCB 126 was anticipated to cause significant induction of CYP1A in this study due to its high affinity for the Ah receptor. Results showed that CYP1A induction followed similar trends in both medaka age groups, i.e., only the highest PCB dose resulted in significant hepatic enzyme induction. As little as 3 d post-PCB exposure, CYP1A activity was apparent. This observation is consistent with that reported for other teleost species. For example, channel catfish (*Ictalurus punctatus*) injected i.p. with the same dose of PCB 126 showed significant hepatic CYP1A induction at 3 d post-exposure (Rice and Schlenk, 1995; Rice and Roszell, 1998).

Although greatest enzyme induction in medaka was observed 14 d post-exposure in both age groups treated with the highest PCB dose, the lower PCB dose failed to have a significant effect. This observation appears to contrast that observed in channel catfish exposed via the same route and at the same PCB 126 doses used in this study. In the latter study, significant CYP1A induction was apparent 7 d post-exposure in both the low and high PCB-treated catfish (Rice and Roszell, 1998). Since medaka were only observed in the present study at 3 and 14 d post-PCB injection, it may have been possible that significant CYP1A induction also occurred in medaka after 7 d. European flounder (*Platichthys flesus*) treated orally with PCB 126 at concentrations ranging from 0.5 – 50 mg/kg demonstrated significant induction of CYP1A (compared to controls) up to 16 d

following exposure to all doses tested (Grinwis et al., 2001). In light of these observations, it is possible that Japanese medaka may not be as sensitive as channel catfish to this particular PCB 126 dose (0.01 $\mu\text{g/g}$ BW), and/or the persistence of this congener in medaka may not be as great as that observed in flounder (up to 16 d).

Innate Immune Function

Fish age at the time of PCB exposure appeared to be an important factor in determining impact of PCB 126 on innate immune function. While O_2^- production by PMA-stimulated phagocytes from aged fish was only affected 3 d post-injection with the lowest PCB concentration, PMA-stimulated O_2^- production in PCB-treated juvenile medaka was significantly affected at both post-exposure timepoints. The observation that stimulated O_2^- production was affected in aged fish only at the lowest PCB dose and at one post-exposure timepoint (i.e. 3 d) questions the credibility of this response. At 3 d post-exposure, aged control fish produced unexpectedly low quantities of O_2^- , less than half of that produced by juvenile control fish. Had the controls produced greater amounts of O_2^- there would have been no statistical difference between controls and 0.01 μg PCB/g – treated fish. Depressed O_2^- production 3 d post-PCB exposure suggests that juvenile fish were immunosuppressed following PCB exposure. Similar suppression has been reported in channel catfish exposed to PCB 126 at the same concentrations used in this study (Rice and Schlenk, 1995). In the latter study, both PCB doses caused significant depressions in O_2^- production in catfish 7 d post-injection; catfish examined at 3 d post-PCB injection also exhibited impaired O_2^- production, but only following treatment with the highest PCB dose. A recent study by Regala et al. (2001) demonstrated that i.p.

injection of PCB 126 (at the same concentrations used in the current study) significantly suppressed oxidative burst activity by channel catfish phagocytes 14 d post-exposure. Based upon previous literature that demonstrated innate immune suppression in channel catfish 7 and 14 d post-PCB 126 exposure (Rice and Schlenk, 1995; Regala et al., 2001), a similar effect on oxidative burst activity might have been anticipated in Japanese medaka. Instead, at 14 d post-exposure an increase in oxidative burst activity was observed. Considering that the exposure route, PCB dose, and particular congener used in the catfish studies were the same as those employed herein, species variability may account for the differences in oxidative burst activity between PCB-exposed medaka and catfish. Support for this notion comes from the previously-cited differences between medaka and catfish response to PCB-induced CYP1A induction.

Although the increase in phagocytic oxidative burst activity observed in this study 14 d post-exposure appears inconsistent with observations from other fish studies (Regala et al., 2001), similar results demonstrating increased $O_2^{\cdot -}$ production following PCB exposure have been reported in mammals (Ganey et al., 1993; Narayanan et al., 1998). For example, rat PMNs exposed *in vitro* to Aroclor 1242 demonstrated increased respiratory burst activity (Ganey et al., 1993). Investigators suggested that PCBs may have either directly affected protein kinase C (PKC, which upon activation is associated with $O_2^{\cdot -}$ production in PMA-stimulated PMNs) or increased the rate of $O_2^{\cdot -}$ production by an alternative mechanism (Ganey et al., 1993). Another explanation for the observed increase in intracellular $O_2^{\cdot -}$ production following PCB-126 exposure in juvenile fish is that this congener may inhibit the enzymatic activity of superoxide dismutase (SOD)

(Narayanan et al., 1998). Superoxide dismutase catalyzes the formation of hydrogen peroxide (H_2O_2) from two molecules of O_2^- . Inhibition of this enzyme could lead to increased concentrations of O_2^- within the cell. Support for this hypothesis comes from a study in a cell-free system demonstrating the ability of PCBs to inhibit SOD activity (Narayanan et al., 1998). In this study, authors demonstrated SOD inhibition by Aroclor 1242 (1 $\mu\text{M}/\text{ml}$) and PCB 47 (1 $\mu\text{M}/\text{ml}$). Reaction mixtures containing either Aroclor 1242, PCB 47, or diethyldithiocarbamate (DDC, an inhibitor of SOD) were incubated with bovine copper-zinc SOD for 30 min and the autoxidation of 6-hydroxydopamine (6-OHDA) was measured spectrophotometrically. In the presence of SOD, autoxidation of 6-OHDA was inhibited because SOD scavenged any O_2^- present. Results from this *in vitro* study demonstrated that incubation with Aroclor 1242, PCB 47 or DDC increased the autoxidation of 6-OHDA, indicating that all of these compounds inhibited SOD activity (Narayanan et al., 1998). Further evidence for PCB-induced effects upon SOD comes from studies examining feral populations of smallmouth bass (*Micropterus dolomieu*) residing in PCB-contaminated environments (Anderson et al., 1997). In these studies, fish from the PCB-impacted site had reduced splenic and hepatic SOD activity compared to fish collected from a reference site.

The finding that PMA-stimulated O_2^- production in aged medaka was not altered by PCB exposure suggests that juvenile fish may be more sensitive than older fish to PCB-induced effects upon the innate immune response. Although this finding contradicts our original hypothesis that aged fish were more susceptible to PCB-induced immunotoxicity than juvenile fish, it has been noted for mammalian species that sensitivity to toxic insult

decreases as age increases (i.e., adults are less sensitive than juveniles) (Parkinson and Safe, 1987). Thus, it is possible that this may also be true for fish species.

The significant increase in unstimulated $O_2^{\cdot -}$ production observed in both age groups 14 d post-PCB injection indicates that PCB 126 may be acting as an oxidant. Similar observations have been made in mammals, whereby quiescent cells exposed *in vitro* to PCBs significantly increased $O_2^{\cdot -}$ production (Ganey et al., 1993; Narayanan et al., 1998). Incubation of human neutrophils for only 30 min with either Aroclor 1242 or PCB 47 (at 10 $\mu\text{g/ml}$) was sufficient to increase $O_2^{\cdot -}$ production (Narayanan et al., 1998). In a similar study, rat PMNs incubated *in vitro* for 30 min with Aroclor 1242 (at 10 $\mu\text{g/ml}$) also demonstrated significant increases in $O_2^{\cdot -}$ (Ganey et al., 1993). It is possible that PCB-induced inhibition of SOD is responsible for the increase in $O_2^{\cdot -}$ production observed in unstimulated cells, as well as in PMA-stimulated cells.

An alternative explanation for the increased levels of $O_2^{\cdot -}$ measured in PCB 126-exposed medaka may involve reduction of the cellular antioxidant enzyme glutathione peroxidase (GPX). As an antioxidant enzyme, GPX protects cells against lipid peroxidation and oxidative stress (Twaroski et al., 2001). It has previously been reported in mammals that PCBs, particularly those that activate the Ah receptor, can lead to depressed GPX activity (Jin et al., 2001; Twaroski et al., 2001). For example, rats that received two i.p. injections (3 d apart) of the coplanar PCB 77 (100 $\mu\text{mol/kg/injection}$) and examined 1, 2 or 3 weeks later demonstrated depressed total GPX and selenium-dependent GPX (Se-GPX) levels at all post-injection timepoints (Twaroski et al., 2001). Furthermore, white

Leghorn chicken (*Gallus domesticus*) embryos injected with PCB 126 (1.2 and 1.6 µg/kg egg) and examined 19 d later demonstrated depressed liver GPX activity (Jin et al., 2001). This data offers another possible mechanism by which PCB 126 may be acting to induce oxyradical stress in Japanese medaka.

Metabolism of PCB 126 may have contributed to the observed differences in $O_2^{\cdot -}$ production by juvenile and aged fish. While both age groups exhibited substantial CYP1A induction 14 d following exposure to the highest PCB concentration, enzyme induction in the juvenile fish exceeded that of the older fish. It is well-documented that coplanar PCBs are strong Ah receptor agonists and inducers of cytochrome P-450 isozymes (Schlezniger et al., 1999). Induction of these enzymes can lead to PCB metabolism and the formation of oxidized metabolites that can lead to the formation of reactive oxygen intermediates (ROI) (Tworoski et al., 2001). In light of this, it is possible that greater CYP1A induction by juvenile medaka was responsible for creating increased amounts of oxidized PCB 126 metabolites and subsequent ROI production. This is supported by the fact that greater enzyme induction was apparent after 14 d, which corresponds to the time when ROI production was enhanced in juvenile fish.

Humoral Immune Function

Suppression of the humoral immune response has been observed in various species following exposure to PCBs. The PFC response to a T-dependent antigen (i.e. SRBCs) has been reported to be an extremely sensitive indicator of polyhalogenated aromatic hydrocarbon (PHAH) toxicity in mammalian species (Lubet et al., 1986). Likewise,

similar responses were observed in this study in both juvenile and aged Japanese medaka following PCB exposure.

The PFC response requires the cooperation of all three arms of the immune system (i.e. innate, cellular, and humoral-mediated). Therefore, disruption of any one component could ultimately lead to an altered PFC response. The observation that humoral, but not innate, immune function was impacted by PCB-exposure in aged fish indicates that T- and/or B-cells, rather than macrophage, were involved in the impaired PFC response of Japanese medaka. This is consistent with reports suggesting that humoral immune suppression in PHAH-exposed mice is most likely the result of effects upon lymphocytes (Lubet et al., 1986). A depressed PFC response was apparent in aged fish treated with the highest PCB concentration only; this response was magnified as post-exposure duration increased. This suggests that the mechanism(s) responsible for producing a toxic outcome was (were) unable to recover from the initial PCB exposure.

The observation that juvenile and aged fish exhibited similar PFC responses 3 d after exposure to the highest PCB concentration indicates that host age at the time of exposure does not influence the effects of PCB on PFC numbers. However, as post-exposure time increased to 14 d, juvenile fish responded to a PCB concentration lower than shown to produce effects in aged fish. Results further demonstrated that macrophage function in juvenile medaka was particularly sensitive to the effects of PCB 126 exposure. Therefore, the ability of PCBs to alter both innate and humoral immune responses of juvenile fish may contribute to the enhanced sensitivity of the juvenile fish.

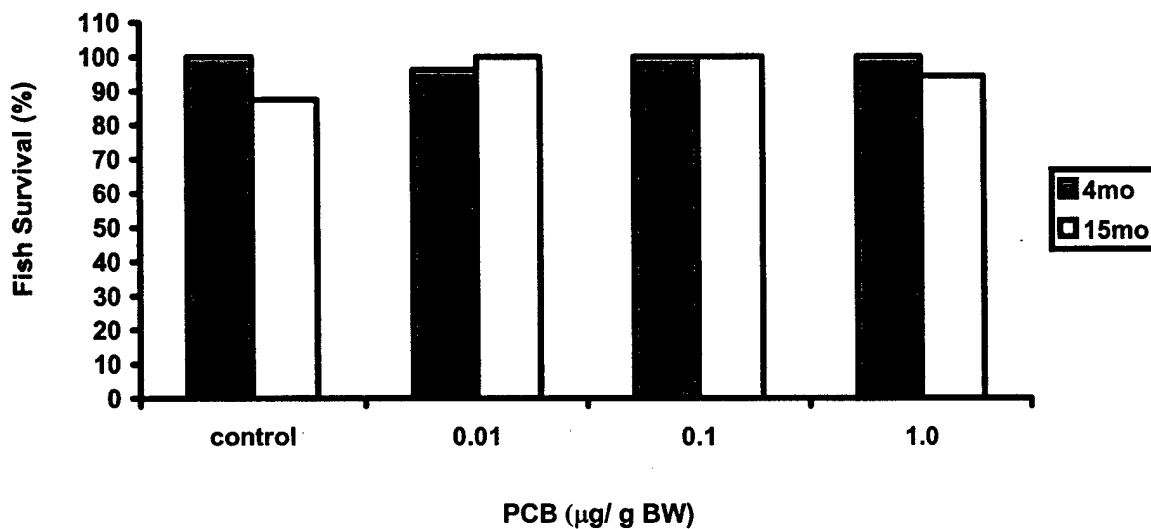
Recovery from a suppressed PFC response has been observed in mice examined 6 or 16 weeks following a single injection of 500 mg/kg Aroclor 1254 (Lubet, et al., 1986). It may be possible that fish have a similar potential for recovery. The observation that juvenile medaka did not experience further immune suppression 14 d after a single PCB exposure suggests that fish may have recovered from the toxic insult. However, the same does not appear to be true for aged medaka. Considering that 15 – mo-old fish may have been immunocompromised prior to PCB exposure, inability to recover would be expected. Taken together, it appears likely that the differences observed between juvenile and aged medaka is due to the underlying cellular mechanism(s) involved in bringing about the PFC response.

Conclusions:

This study identifies both host age and post-exposure duration as factors influencing the impact of PCB 126 on the fish immune system. Furthermore, results demonstrated that the Japanese medaka is a sensitive laboratory model for determining PCB-induced immunodysfunction. As has been observed for other fish species and mammalian models, O_2^- production and the PFC response appear to be sensitive endpoints for determining the immunotoxic effects of PCB 126 in Japanese medaka. Further investigations are warranted regarding the cellular mechanism(s) by which host age may affect PCB-induced immunotoxicity. Moreover, closer examination of the relationship between PCB exposure and antioxidant enzymes, drug-metabolizing enzymes, and ROI production may lead to a better understanding of the observed results. Taken together, these studies demonstrate the sensitivity of the fish immune response for assessing the persistent immunotoxic effects of a ubiquitous, coplanar PCB congener in contaminated aquatic environments.

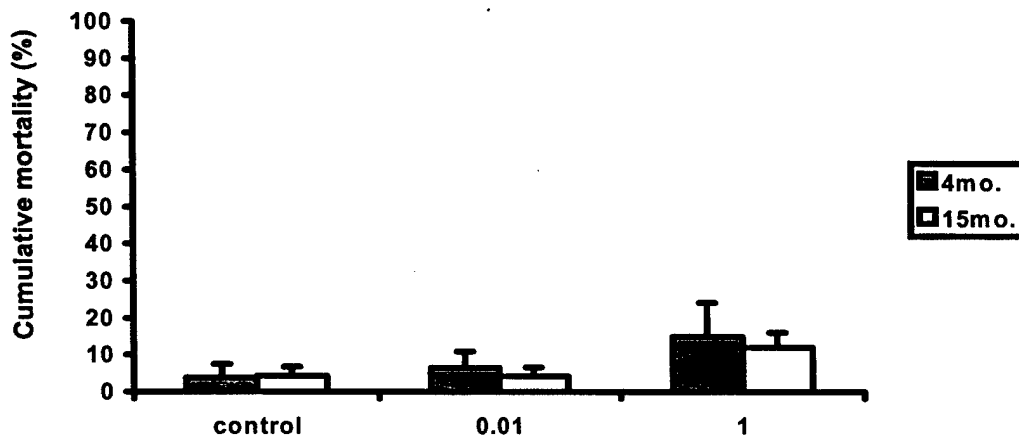
Figure 1

Percent Survival For Up to 14 d Post-Injection with PCB 126^a



^a Values from pilot studies completed for each age group (n=1)

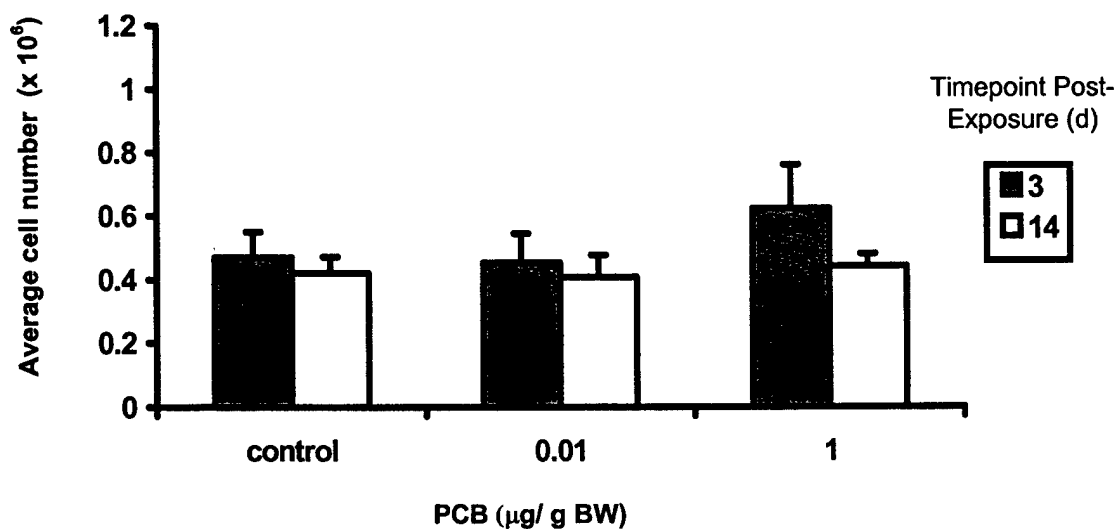
Figure 2



^a Each bar represents the mean (n=13 experiments) ± SEM

Figure 3A

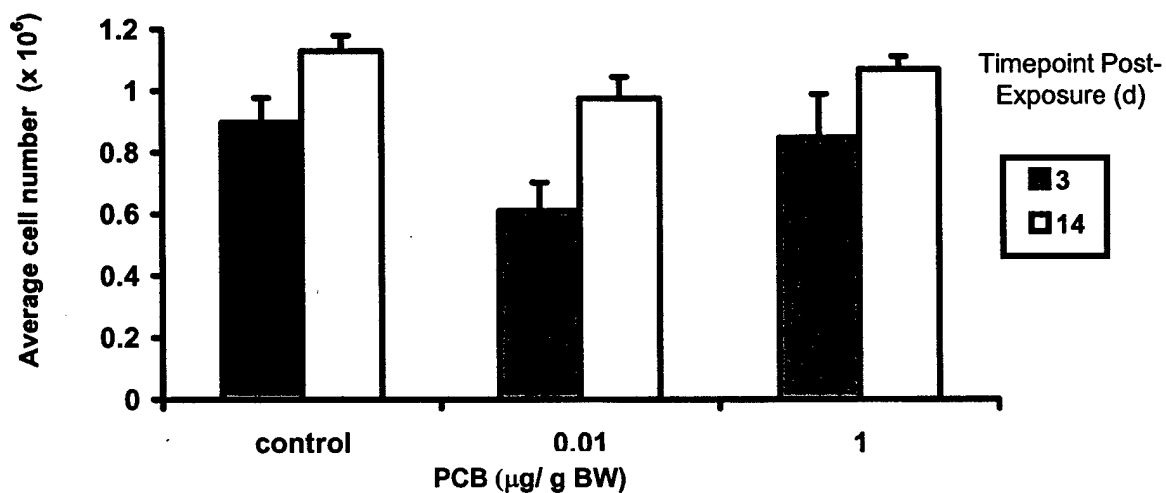
Kidney Cell Numbers in Juvenile Medaka^a



^a Each bar represents the mean (n=9-10 experiments) +/- SEM

Figure 3B

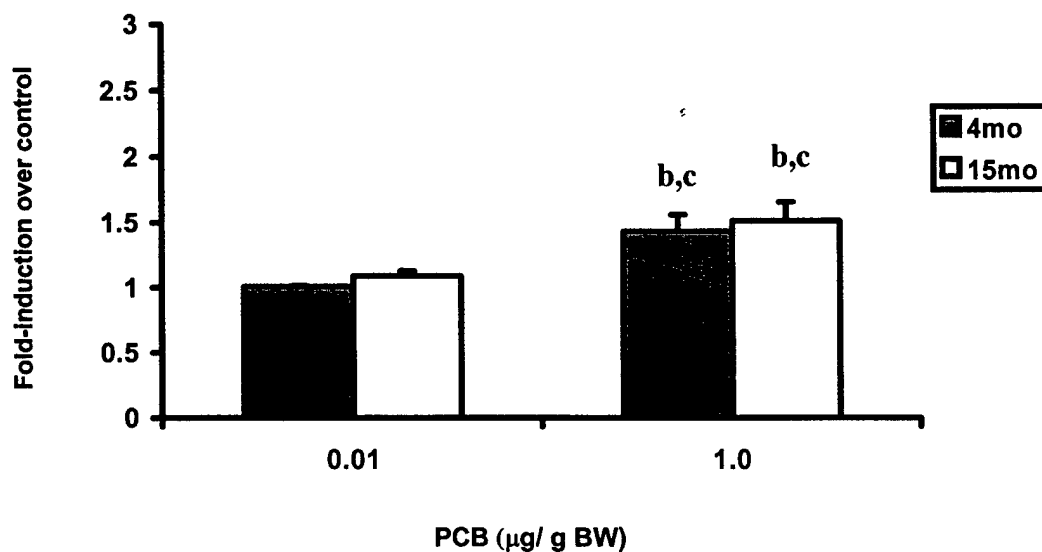
Kidney Cell Numbers in Aged Medaka^a



^a Each bar represents the mean (n=7 experiments) +/- SEM

Figure 4A

CYP1A Enzyme Induction 3 d Post-PCB Exposure^a



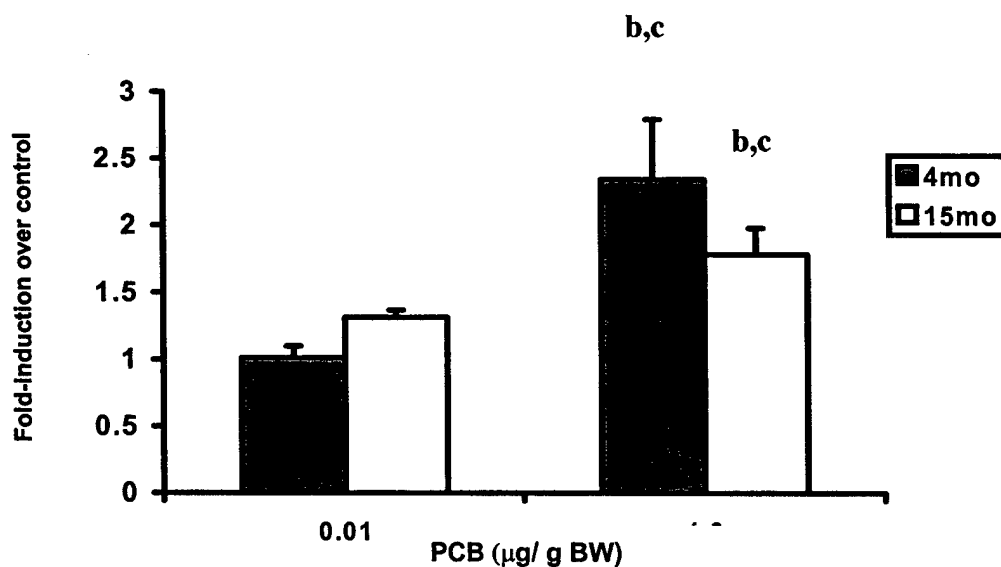
^a Each bar represents the mean (n=2-4 experiments) \pm SEM

^b Significantly different ($p < 0.05$) from age-matched control

^c Significantly different ($p < 0.05$) from 0.01 µg/g BW group

Figure 4B

CYP1A Enzyme Induction 14 d Post-PCB Exposure^a

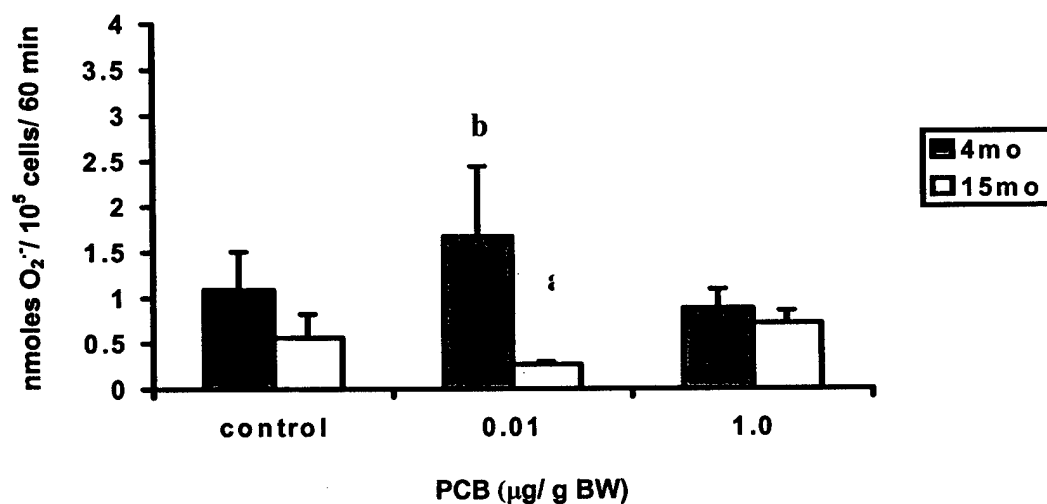


^a Each bar represents the mean (n=2-4 experiments) \pm SEM

^b Significantly different ($p < 0.05$) from age-matched control

^c Significantly different ($p < 0.05$) from 0.01 µg/g BW group

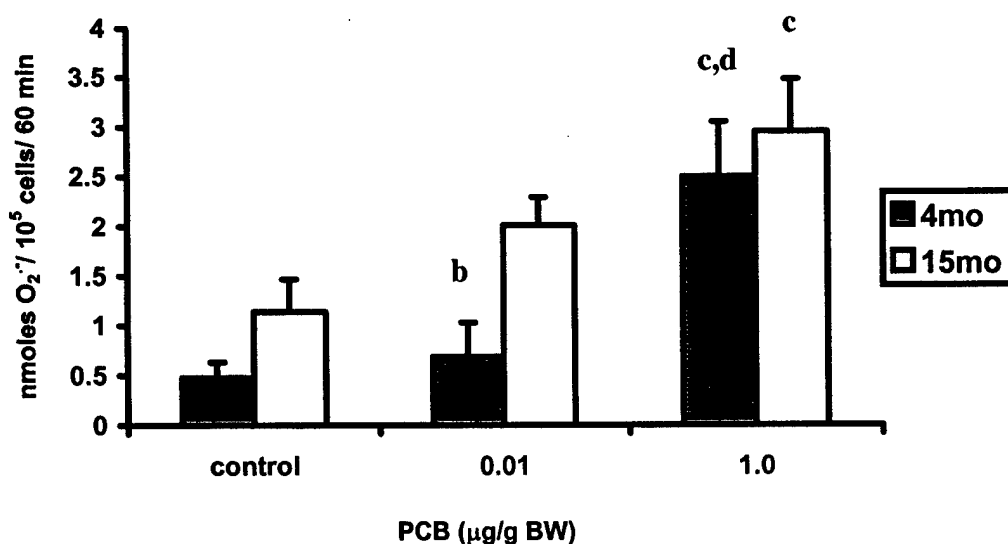
Figure 5A
Unstimulated O_2^- Production 3 d Post-PCB Exposure^a



^a Each bar represents the mean (n=3-4 experiments) \pm SEM

^b Significantly different ($p < 0.05$) from 15-mo-old fish within a single exposure group

Figure 5B
Unstimulated O_2^- Production 14 d Post-PCB Exposure^a



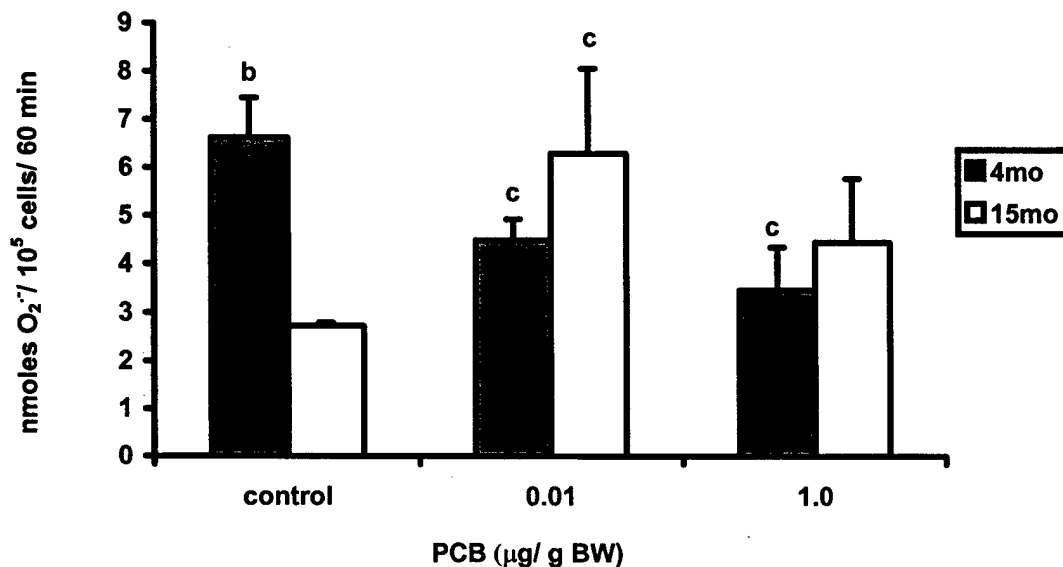
^a Each bar represents the mean (n=3-4 experiments) \pm SEM

^b Significantly different ($p < 0.05$) from 15-mo-old fish within a single exposure group

^c Significantly different ($p < 0.05$) from age-matched control

^d Significantly different ($p < 0.05$) from 0.01 µg/g BW group

Figure 6A
Stimulated O_2^- Production 3 d Post-PCB Exposure^a

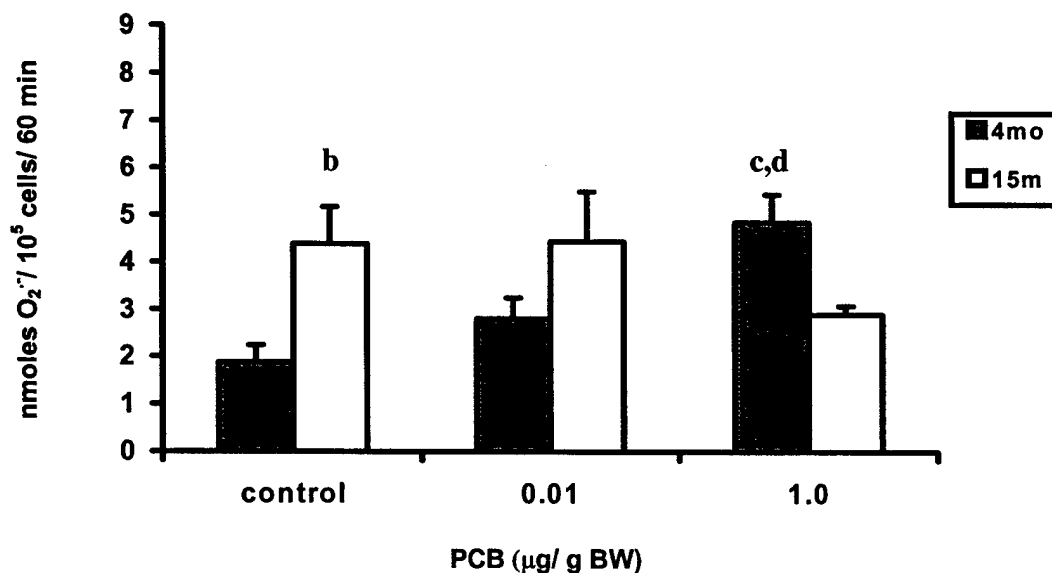


^a Each bar represents the mean (n=3-4 experiments) +/- SEM

^b Significantly different (p < 0.05) from 15-mo-old fish within a single exposure group

^c Significantly different (p < 0.05) from age-matched control

Figure 6B
Stimulated O_2^- Production 14 d Post-PCB Exposure^a



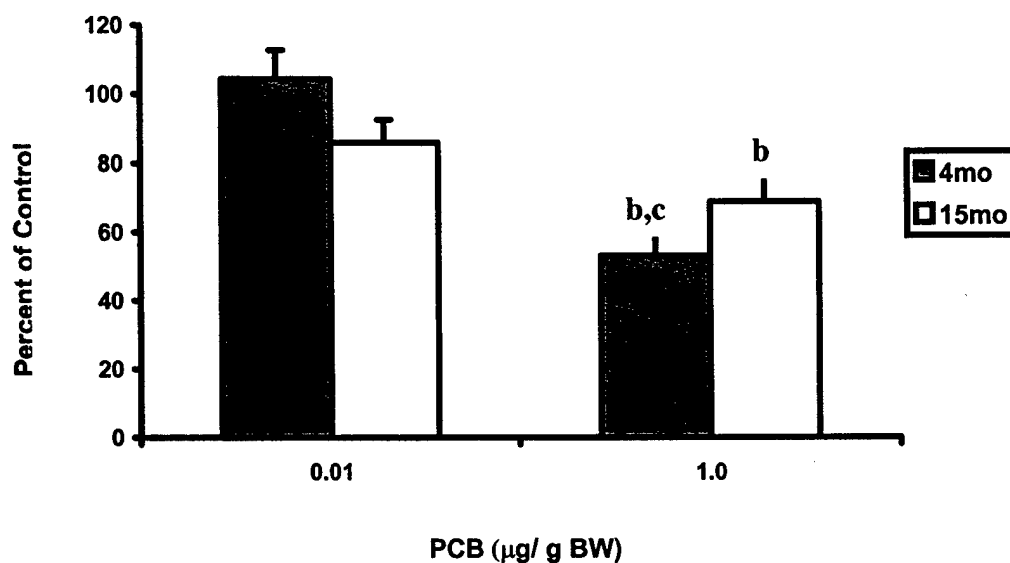
^a Each bar represents the mean (n=3-4 experiments) +/- SEM

^b Significantly different (p < 0.05) from 4-mo-old fish

^c Significantly different (p < 0.05) from age-matched vehicle control

^d Significantly different (p < 0.05) from 0.01 µg/g BW group

Figure 7A
PFC Response 3 d Post-PCB Exposure^a

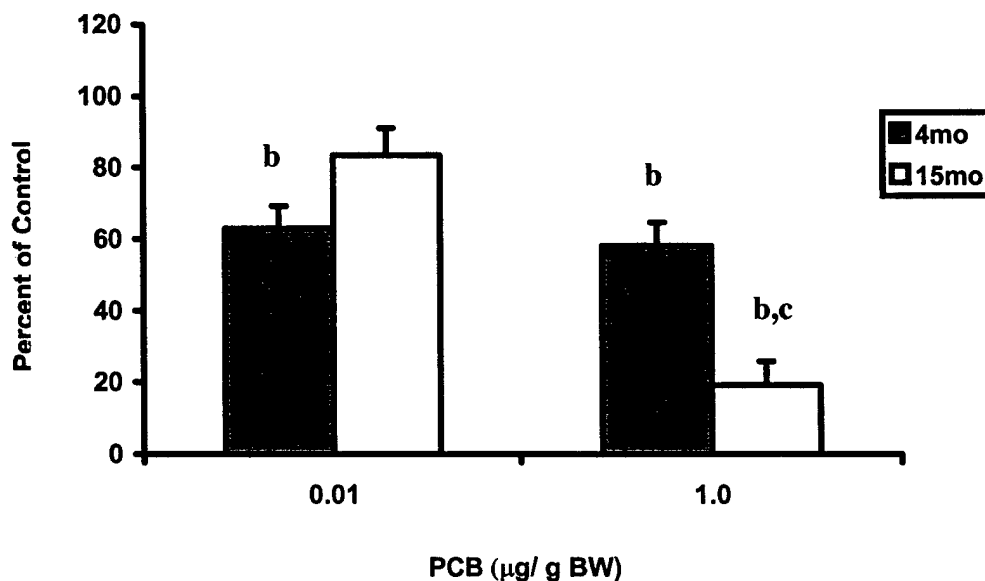


^a Each bar represents the mean (n=3-4 experiments) +/- SEM

^b Significantly different (p < 0.05) from age-matched control

^c Significantly different (p < 0.05) from 0.01 µg/g BW group

Figure 7B
PFC Response 14 d Post-PCB Exposure^a



^a Each bar represents the mean (n=3-4 experiments) +/- SEM

^b Significantly different (p < 0.05) from age-matched control

^c Significantly different (p < 0.05) from 0.01 µg/g BW group

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